Isolation, characterization and metal response of novel, acid-tolerant *Penicillium* spp. from extremely metal-rich waters at a mining site in Transbaikal (Siberia, Russia)

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ABSTRACT

The role of fungi in metal cycling in acidic environments has been little explored to date. In this study, two acid-tolerant and metal-resistant *Penicillium* strains were isolated from a mine site in the Transbaikal area of Siberia (Russia). Waters at the mine site were characterized by extremely high metal concentrations: up to 18 g/l iron and more than 2 g/l of each of copper, zinc, aluminum, and arsenic. Both strains were identified as *Penicillium* spp. by phylogenetic analyses and they grew well in Czapek medium acidified to pH 2.5. Resistance to Cu, Cd, Ni, Co and arsenate was in the range of 1-10 g/l. Further experiments with one isolate demonstrated that growth in Cu-containing media was accompanied by the precipitation of Cu-oxalate (moolooite) and the formation of extracellular vesicles enriched in copper on mycelia. Vesicles were reduced in size by 2-3 orders of magnitude in Cd-containing media and were not formed in the presence of Ni or Co. Cd-oxalate was detected as a crystalline solid phase in Cd-exposed mycelia. Hydrated Ni-sulfate (retgersite) and Co-sulfate (bieberite) were detected in mycelia grown in the presence of Ni and Co, respectively. The results demonstrated that acid-tolerant and metal-resistant *Penicillium* constitute a component of extremophilic microbiomes, contributing to organic matter breakdown and formation of secondary solid phases at pH ranges found in acid rock drainage.

INTRODUCTION

Waste streams from mining and mineral processing are typically characterized by elevated concentrations of metals, sulfate and, often, low pH. The main types of waste from sulfide mining include gangue material, uneconomic minerals associated with ores, and flotation tailings. The latter are typically very fine material produced by ore milling and extracting valuable metals by hydrometallurgy. The residual sulfide-rich ore tailings are often disposed at the mining site anmay be subject to oxidation processes, which release protons, sulfate, iron and other metal ions. These aggressive waters originating from mine waste streams are designated acid mine drainage (AMD) or acid rock drainage. The metal concentration in AMD is dependent on the residual sulfide content of the mine wastes, exposed surface areas, grain sizes, access to oxygen, type of sulfide, microbiological processes and bulk hydrogeological and geochemical environment. This study focuses on an especially aggressive AMD at the Sherlovaya Gora polymetallic deposit in the Chita region, Russia, with unusually high metal and arsenic concentration. During Soviet times, much of the high grade metal sulfide ore was not sent to processing, but deposited and abandoned on the site along with mining waste. Exposure to oxygen and precipitation resulted in formation of acidic mine leachate containing extremely high concentrations of iron, copper, zinc, arsenic, and other toxic metal and metalloids.

Hitherto, emphasis on examining microorganisms in AMD has focused on prokaryotic biology (e.g., Johnson and Hallberg, 2008; Bonnefoy and Holmes, 2012; Ilbert and Bonnefoy, 2013; Jerez, 2013; Mendez-Garcia et al., 2015). Only in recent decades have eukaryotic microorganisms been investigated in mining-impacted habitats, with the Richmond Mine in northern California (Baker, Banfield 2003; Baker et al., 2004; Baker et al., 2009),and Rio Tinto (Amaral Zettler et al., 2002; Aguilera et al., 2006; Aguilera et al., 2007; Aguilera, 2013) being a notable examples. All of the above-mentioned studies used molecular techniques to demonstrate the presence of fungi in AMD. The sporadic reports of fungal occurrence in mine-associated ecosystems were also chiefly based on the 18S rRNA or ITS signatures. Thus, ascomycota, chytridiomycota, and zygiomycota were observed in the Konigstein underground uranium mine (Zirnstein et al., 2012). *Penicillium* clones have been retrieved from pyrite ore tailings in China (Hao et al., 2010). A recent study revealed *Ascomycota*, *Chytridiomycota*, *Basidiomycota*, *Nuclemycea*, and LKM15 in biofilms from an abandoned underground mercury mine in Spain by high-throughput 18S rRNA gene sequencing (Mesa et al., 2017).

A few cultivated isolates have been obtained from AMD-associated environments. *Purpurecillium liliacinum*, *Bispora* sp. and *Penicillium* spp. were isolated from Rio Tinto

(Oggerin et al., 2013). Several acid-tolerant and acidophilic fungi were isolated from acidic soils associated with a kaolin quarry with brown coal bed, including *Acidiella bohemica* (Hujslova et al., 2013), *Acidothrix acidophila*, *Acidea extrema*, and *Soosiella minima* (Hujslova et al., 2014). An acid-tolerant *Hortaea acidophila* originated from lignite particles (Holker et al., 2004). Different strains of *Acidomyces acidophilum* were isolated from elemental sulfur or pyrite ores (Selbmann et al., 2008). Members of the above-mentioned species of melanised fungi have been also isolated from acidic springs (Hujslova et al., 2013). This type of habitat also harbored acidophilic *Teratosphaeria acidotherma*, later reclassified as *Acidomycesacidotherma* (Yamazaki et al., 2010).

The biogeochemical role of fungi in mining-impacted environments is not well understood and the information is fragmentary (Baker and Banfield, 2003). Prokaryotes are considered to be the major players in element cycling in AMD (Mendez-Garcia et al., 2015). However, as shown with prokaryotes, fungi can also modify their environment and cause changes in aqueous solution and solid phases. For example, *Aspergillus niger* has been shown to solubilize cuprite (CuO_2), galena (PbS), rhodochrosite ($Mn(CO_3)_x$), and calcite ($CaCO_3$) (Sayer et al., 1997). *Beauveria caledonica* (Basidiomycetes) was shown to form uranium-containing precipitates on gill surfaces (Gadd, 2007). *B. caledonica* precipitated oxalates in presence Cd^{2+}, Cu^{2+} and Zn^{2+} (Fomina et al., 2005). *Penicillium simplicissimum* has been shown to precipitate various metal oxalates (Fomina et al., 2005; Gadd, 2007). Liang et al. (2016) demonstrated the formation of Pb-oxalate and Pb-pyromorphite by *Aspergillus niger* and *Paecilomyces* via reactions whereby phosphatase released inorganic phosphate from phytic acid or glycerol-2-phoshate, precipitating lead that had been added as Pb-nitrate to the assay. Pyromorphite is metastable and can be converted to Pb-oxalate in fungal cultures when oxalic acid is the major metabolite (Fomina et al., 2004). The formation of insoluble or poorly soluble metal oxalates changes the bioavailability of potentially toxic metals.

In the present study, the isolation is reported of two novel *Penicillium* strains that could tolerate high concentration of Cu, Cd, and As and low pH, from the aggressive AMD environment of Sherlovaya Gora mine, Russia. The possible biogeochemical role of these isolates is related to changes in metal solubility as well as ultrastructural and morphological responses to elevated metal concentration.

MATERIALS AND METHODS

Sample site. Sherlovaya Gora [Шерловая Гора], literally translating as "Schorl Mountain", is a polymetallic opencast mine site, containing extensive spoils and tailings areas. The site occupies a cluster of hills, among the plains of the Onon and Borzya Rivers, in the Transbaikal area (or Zabaikalsky krai), 250 km SE of Chita, the capital city of the region. The Sherlovaya Gora hills can be subdivided into a greisenised Jurassic granitic dome (associated with commercial occurrences of topaz, beryl, tourmaline, bismuth and tungsten) and a probable Early Cretaceous eruptive/subvolcanic complex, hosting the main cassiterite and polymetallic sulphide orebodies, and recently worked via a large opencast (Kokunin 2006; Kozlov 2009; Klopotow & Kantor 2014; Banks et al. 2015). The main sulphide minerals present are pyrite (FeS_2), pyrrhotite, arsenopyrite (FeAsS), galena (PbS), sphalerite (ZnS) and chalcopyrite ($CuFeS_2$). The opencast mine was closed in c. 1995.

Sampling, field measurement, water and sediment analyses The ShG4 sample was collected on 14th July 2013 from a small puddle at the foot of a mine waste stockpile, presumably containing high grade ore (Figure 1).The upper layer of the sediment was grab sampled to sterile polyethylene tubes for microbiological and mineralogical analysis. Water for chemical analysis was filtered using a Millipore filter with 0.45μm pore size. Prior to analysis, samples were stored at 4°C. Redox potential, pH, and temperature were measured on-site by inserting electrodes (Hanna Instruments HI8314F) into the water-saturated sediments. The elemental composition of the ShG4 water sample was analyzed by ICP-MS by the Plasma Chemical-Analytical Center (Tomsk). The mineralogical composition of the ShG4 sediment sample was characterized by X-ray diffraction (XRD) using a Shimadzu XRD-6000 diffractometer as previously described by Ikkert et al. (2013). Air dried sediments were examined under a Philips SEM 515 scanning electron microscope (SEM). Energy dispersive spectrometry (EDS - using an EDAX Inc, Mahwan USA spectrometer) was performed at a voltage of 30 kV and a working distance of 12 mm.

Enrichment and isolation of fungi. Microbiological analyses were initiated by preparing enrichments in Widdel and Bak medium (Widdel and Bak, 1992) containing:….(pH 2.5) from the ShG4 sediment sample. Culture tubes contained 5 ml medium and were stoppered with aluminum caps and incubated at 28°C for 2 days. Subsequent cultures were grown in modified Czapek medium (per liter: 30 g glucose; 40 g yeast extract; 3 g $NaNO_3$; 1 g K_2HPO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 g KCl; 0.01 g $FeSO_4 \cdot 7H_2O$). The medium was acidified to pH 2.5 with HCl and further modified by the addition of 3 g l^{-1} Na_3AsO_4 and 0.055 g l^{-1} of $CdCl_2$ in all subsequent

experiments unless otherwise stated. Arsenate and cadmium were added to increase the selection pressure in the enrichment phase. Cultures were repeatedly purified through serial dilutions of spores from agar-solidified modified Czapek plates with only a single colony. The isolates were also grown on standard potato dextrose agar and malt extract agar to observe colonial morphology. Bacterial contamination was ruled out by the lack of PCR products with universal bacterial 16S rRNA gene primers. Microscopic examination revealed no bacterial cells.

Growth experiments. Growth at different pH values was measured in 5 ml aliquots of Czapek liquid medium, inoculated with 10 mg wet-weight biomass and amended with 3 g l^{-1} Na_3AsO_4 and 0.055 g l^{-1} of $CdCl_2$. Cultures were incubated under static conditions for 17 days at 28 °C, followed by harvesting of the the mycelium for dry weight determination. Changes in pH were measured with a model HI 83141 pH meter (Hanna Instruments).

Static cultures were screened in unmodified Czapek media (pH 2.5) for tolerance to arsenate (Na_2HAsO_4), cadmium ($CdCl_2 \cdot 2.5H_2O$), cobalt ($CoCl_2 \cdot 6H_2O$), copper ($CuSO_4 \cdot 5H_2O$), nickel ($NiCl_2$), and lead ($(CH_3COO)_2Pb$) at 28 °C. The metals were tested at 1, 2, 3, 4, 6 and 10 g l^{-1}.

Scanning electron microscopy and energy dispersive analysis of X-rays. Fungal biomass was harvested by centrifugation (13,100 *g*, 10 min, 15 °C). The pellet was washed with 2% oxalic acid (10 min) followed by two washes with distilled water (10 min each) to remove any loosely associated and unsequestered metals. The samples were air-dried, coated with gold, and examined under a model Philips SEM 515 scanning electron microscope equipped with a microprobe (acceleration voltage 30 kV, focal distance 12 mm, and probe size 50-100 nm^2).

X-ray diffraction. Powder X-ray diffraction was performed with a Rigaku Ultima 4 diffractometer (Rigaku Corp., Tokyo) with CuKα radiation. The samples were packed into zero background quartz sample holders and step-scanned from 10 to 75° 2θ using a step interval of 0.02° 2θ and a counting time of 0.8 s. The diffraction patterns were analyzed with Crystallographica-Search Match software and the PDF-4 database (International Center Diffraction Data, http://www.icdd.com).

DNA Isolation and PCR amplification. For identification of the two pure cultures of fungi, mycelia were washed with 2% EDTA in PBS to remove residual metals before DNA was isolated using an MO BIO PowerSoil DNA Kit (MO BIO Laboratories) according to the manufacturer's instructions. Identification was based on PCR amplification of the 18S rRNA, ITS, Tsr1, Cct8, RPB1, and RPB2 genes. PCR primers (Table 1) were synthesized by Syntol (Moscow). The PCR reactions for each primer set were setup in a 25 µl master-mix, using

Thermo Scientific *Taq* and reagents. The PCR program was setup on a Thermo Cyclers Bio-Rad My Cycler™ and T100™ as follows: 30 cycles of 30 s at 94 °C; 30 s primer annealing; 1 min at 72 °C and final extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gels stained with ethidium bromide. The PCR products were sequenced at the Bioengineering Centre of the Institution of the Russian Academy of Sciences (Moscow). The closest related species were identified using Basic Local Alignment Search Tool (BLAST - http://www.ncbi.nlm.nih.gov/blast/). A phylogenetic tree was constructed with concatenate sequences of genes RPB1, RPB2, Cct8 and Tsr1 with maximum likelihood using MEGA6 software (Tamura et al., 2013). Concatenation was based on Houbraken and Samson (2011). The robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations.

RESULTS

Physicochemical characteristics of the sampling site. The water collected at the sampling site ShG4 had an intense red colour and was characterized by an extremely low pH=1.95, a temperature (in July) of 9.5°C and oxidized conditions (Eh = + 689 mV). The water sample contained extreme concentrations of many metals, including 18.3 g/l iron, 2.1 g/l copper, 2.8 g/l zinc, and 2.7 g/l aluminum (Figure 2). Arsenic was present at a remarkable concentration of 2.9 g/l. Moreover, very high concentrations of rare earth and radioactive elements suggest extensive leaching of the ore-bearing rocks; for example, thorium was recorded at 6.4 mg/l, uranium 9.2 mg/l, Y – 23.1 mg/ l, Gd – 3.1 mg/l, Dy 5.1 mg/l, and Yb 2.7 mg/l. The mineralogical composition of the sediment sample revealed different U- and Pb-containing secondary phases, namely barysilite, $Pb_8Mn^{II}[Si_2O_7]_3$, magnesiozippeite, $Mg[(UO_2)_2O_2(SO_4)](H_2O)_{3.5}$, renardite, $Pb(UO_2)_4(PO_4)_2(OH)_4{\cdot}7H_2O$, sklodowskite, $Mg(UO_2)_2(HSiO_4)_2{\cdot}5H_2O$, and vanuralite, $Al(UO_2)_2(VO_4)_2(OH){\cdot}11(H_2O)$ (Figure 2).

Isolation and characteristics of the fungal cultures. The initial enrichments were intended to isolate sulfidogenic prokaryotes; however, two fungal cultures developed and overgrew bacteria in the Widdel and Bak media supplemented with pepton (?) as an electron donor after 3 days of incubation. The cultures were purified by repeated dilutions to extinction in modified Czapek medium, checked for bacterial contaminants, and designated ShG4B and ShG4C. In repeated subcultures, spores of ShG4B germinated after 10 days and those of ShG4C after 3 days. Although not measured, similar differences of several days were noted for growth rates.

Hyphae of both isolates had septate mycelium and paniculate conidiophores. The morphological traits (Figure 3) suggested that the isolates belonged to the genus *Penicillium*.

Based on the 18S rRNA gene sequences, the isolates ShG4B and ShG4C were identified as *P. janthinellum* (99% similarity) and *P. namyslowskii* (100% similarity), respectively. This was corroborated by sequence analysis of the ITS1 and ITS4 regions of rRNA, which exhibited 99% similarity to *P. janthinellum* and 100% to *P. namyslowskii*. Recent phylogenetic studies have defined *Penicillium* as a polyphyletic genus (Houbraken and Samson, 2011; Visagie et al., 2014). To clarify the phylogenetic identity further, several housekeeping genes of the two isolates were amplified and sequenced. Based on the sequences of RPB1, RPB2, Cct8 and Tsr1 genes, the isolates are related at 87% similarity and thus do not represent *P. janthinellum* and *P. namyslowskii.* The phylogenetic position suggests that *Penicillium* sp. ShG4B and *Penicillium* sp. ShG4C are novel species, related to *P. janthinellum* and *P. namyslowskii*, respectively (Figure 4). Further phylogenetic and taxonomic description of these isolates was not within the scope of the study.

Penicillium sp. ShG4C was able to grow in the presence of up to 10 g Cu or Cd/l and 6 g As/l, but was sensitive to Ni (Table 2). *Penicillium* sp. ShG4B was generally not as resistant as isolate ShG4C, but relatively high resistance was noted for As and Co (Table 2). *Penicillium* sp. strain ShG4C formed cleistotecia in presence of copper and cadmium-enriched media (Figure 3).

Both isolates were able to grow over a wide range of pH values from 1.5 to 13 (Figure5). Maximal dry biomass, measured after 17 days of incubation, was formed by ShG4B at initial pH 2.5. Strain ShG4C produced maximal biomass between initial pH 2.5and 7 (Figure 5). It should be noted that the initially low and initially high pH values converged to pH 7-8 during the incubation of ShG4B. ShG4C, on the other hand, did not change the culture medium pH when grown at pH between 1.5 and 2. When cultivated at pH higher than 2.5, ShG4C increased the pH to 7-8; an initial high pH was also decreased to 7-8. At initial pH >8, mycelia were particularly compact and dense as was also noted for the isolates grown in the presence of 3 g Cd L^{-1}. The time course of pH changes is shown in Figure 6 for the two isolates ShG4B and ShG4C grown at initial pH 2.2 and 2.6, respectively. The initial pH values gradually increased during incubation by 3 to 5 units depending on the isolate and length of incubation and reached circumneutral pH after 12 days of incubation.

Mineral formation in ShG4C cultures. Because isolate ShG4C had a higher tolerance to metals, it was selected for further study of metal (Cu, Cd, Ni, and Co) precipitates. Isolate

ShG4C was grown in the presence of 3 g/l each of Cu, Cd, Ni, and Co at initial pH 2.5 for 17 days to study mineral formation by XRD analysis. Chemical controls without the biomass were included to account for abiogenic chemical precipitation. None of the chemical controls were found to yield crystalline phase precipitates detectable by XRD, with the exception of calcite precipitation in the Cd chemical control (Figure S1). *Penicillium* mycelia grown in the presence of Cu had an XRD pattern consistent with moolooite (Cu-oxalate), suggesting oxalic acid was one of the major metabolites (Figure 7). In the presence of Cd, cadmium oxalate was associated with the mycelia, and no other Cd-containing mineral phases were apparent in the X-ray patterns (Figure 7). Nickel and cobalt in the medium were precipitated in the mycelia as the respective sulfates, retgersite ($NiSO_4 \cdot 6H_2O$) and biebirite ($CoSO_4 \cdot 7H_2O$) (Figure 7).These hydrated sulfates were not detected in the respective heat killed mycelia controls, which suggests that fungus played a role in their formation (Figure 8). XRD patterns of precipitates detected vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) in all samples with heat-killed biomass (Figure 8). In addition, Cu-oxalate (moolooite) was formed in the Cu-amended sample with heat-killed biomass (Figure 8).

Mycelial morphology examined under electron microscopy. Oval-to-round structures were formed on mycelia grown in the presence of Cu. Scanning electron micrographs of strain ShG4C mycelia grown in the presence of Cu for 20 days showed the presence of small round structures that were sometimes in clusters and also elongate (Figure 9). The oval-to-round structures, as well as some larger spherical structures, were attached to the mycelium. They ranged from 0.12 µm to 4 µm. The spores of *Penicillium* sp. ShG4C ranged between 1.2 and 2.2 µm. (То есть это могут быть споры или нет? Какие есть доказательства) Micrographs of mycelia grown with Cu for 40 days showed similar extramycelial structures but the smaller and elongated structures were not present (Figure 9). The elemental composition of the mycelium surface and the vesicular structures showed an elevated level of Cu as compared to that in the mycelium (Figure S2). These extramycelial vesicular attachments were absent in mycelia grown in the absence of metals (Figure 10A). Cobalt sulfate (bieberite) precipitated in needle-shaped crystals, which formed abundant clusters (Figure 10B, C). As shown in Figure S3, Co was detected in the mycelia and particularly in the cluster of crystals. Ni precipitates were not substantiated with scanning electron microscopy, whereas Cd-treated mycelia had small extracellular attachments (50-70 nm) of both square and spherical types (Figure S4). EDXA probing did not detect Cd in the mycelia (Figure S4), although XRD analysis demonstrated the

presence of Cd-oxalate. In the absence of further analytical information, these are attributed to incipient stages of extracellular vesicles that were inhibited by Cd from further development.

DISCUSSION

To the best of our knowledge, the isolation of fungi has not been previously reported from ecosystems characterized by such exceptionally high concentration of metals and metalloids, especially copper and arsenic. Such aggressive environments are rare in the context of typical acid mine drainage (AMD), probably due to a number of factors. Aggressive AMD probably requires a combination of high residual acid-generating sulfide content, lack of buffering mineral capacity, access to oxygen and large exposed surface area, which is seldom encountered. At Sherlovaya Gora. high grade ore has been excavated, left on the surface and exposed to oxygenation and weathering. The metal concentrations found in ShG4 water were several orders of magnitude higher than at other locations associated with mining waste disposal at the Sherlovaya Gora deposit; in other words, the hydrogeochemical environments at the site are very variable (Banks et al. 2015). Although the project team has previously studied microbial communities in the water samples extracted from shallow boreholes drilled into an open cast bench at the Sherlovaya Gora pit, the arsenic concentration did not exceed 49 mg/l and copper did not exceed 81 mg/l (Kadnikov et al., 2016). In the water accumulated in the base of the opencast pit the copper concentration was as low as 3.8 mg/l (Banks et al., 2015). Oxidizing conditions in the studied sediments might be expected to permit the oxidation of tetravalent U to hexavalent uranyl, with the formation of uranyl silicates, such as sklodowskite ($Mg[(UO_2)(SiO_3OH)]_2 \cdot zH_2O$) and other secondary uranium minerals having been detected by XRD analysis. Despite the highly aggressive (and by all conventional perspectives, high toxic) nature of the environment, the sediment still was inhabited by micromycetes.

The noteworthy resistance of strains ShG4B and ShG4C to metals and low pH suggests that these features may reside within separate species. We have reported the full sequence of the mitochondrial genome of strain ShG4C (Mardanov et al., 2016). The phylogenetic analysis of the housekeeping genes performed in this study corroborates the conclusion (based on the phylogenetic analysis of 14 mitochondrial proteins) that strain ShG4C forms a distinct species-level branch within genus *Penicillium*.

The closest relative of *Penicillium* sp. ShG4B was *Penicillium janthinellum*. Recently a Zn-tolerant *Penicillium janthinellum* BC109-2 has been isolated from wetland sediment (Teng et al.,

2017, Archives). The phylogenetic assignment of the strain was performed on the basis of the ITS sequence only, which is not sufficient for resolving differences at the species level in *Penicillium* (reference Houbraken?). Therefore, one cannot exclude the possibility that strain BC109-2 does not belong to *P. janthinellum*. Another *Penicillium janthinellum* strain GXCR, isolated from mining wastes in China, has been reported to tolerate copper at up to 200 mM (12.7 g/l; Feng et al., 2017), which is the same order of magnitude observed for strains ShG4B and ShG4C. However, the methods of taxonomic identification used by the authors are not available. It is conceivable that the isolates from the *Penicillium janthinellum*group (clade?) may possess effective mechanisms to tolerate high metal concentrations. Teng et al. (2017) suggested that extracellular accumulation/precipitation might play a role in the detoxification process in *Penicillium janthinellum* BC109-2, based on the finding of a Zn-enriched cell wall fraction.

The sequestration or compartmentalization of Cu by *Penicillium* ShG4C in our study was associated with the formation of extracellular vesicles, observed in scanning electron micrographs. With Cd, Ni, and Co, the strategy for cellular defense or sequestration is presumed to be different, as extramycelial vesicles were absent. The underlying biochemical and genetic mechanisms of metal resistance associated with extracellular vesicles have not been elucidated in the literature to date. Extracellular vesicles have previously been described in many microscopic fungi (Brown et al., 2015). Their proteomes have revealed a wide array of proteins involved in cell metabolism, signal transduction and virulence as well as structural scaffold proteins and nuclear proteins (Brown et al., 2015). Fungi produce extracellular vesicles (EV) on mycelia, sometimes as part of normal growth or as a stress response depending on the species. Many cellular functions can be associated with EV, implying that vesicles may also serve as an export system. EVs have lipid-bilayers that form lumen-containing spheres, ranging in size from 20 nm to 500 nm in diameter. EVs are formed only in metabolically active cells (Rodrigues et al., 2007, 2014; Wolf et al., 2012). Studies with the plant pathogen *Alternaria infectoria* have shown that EVs harbor virulence proteins (Silva et al., 2014). However, virulence is not the sole function of EVs, as the non-pathogenic yeast *Saccharomyces cerevisiae* produces EVs with at least 400 proteins (Brown et al., 2015). The Cu-enriched extramycelial structures produced by *Penicillium* ShG4C mycelia may represent a stress response to copper. The formation of vesicles in response to Cu in growth medium has not been previously reported and their role has yet to be elucidated.

All acidophilic/acid-tolerant fungal species isolated so far have been classed as melanised fungi. These fungi (known as black yeast, due to their colour), contain melanin in the cell wall,

318 which is believed to protect the cell against harsh environmental conditions (e.g. radiation, heavy
319 metals and oxidizing environments; Hamilton & Gómez, 2002; Gómez & Nosanchuk, 2003).
320 However, the molecular signatures suggested that species close to non-extremophilic thrived in
321 AMD (Baker, from introduction). Both of the strains isolated from Sherlovaya Gora ShG4 could
322 grow in a broad pH range from pH 1.5 through to 13. *Penicillium* ShG4C produced maximal
323 biomass at pH 2.5 and can be considered an acidophilic fungus, while ShG4B can be
324 characterized as acid-tolerant, since it produced maximal biomass in a broad pH range from 2.5
325 through to 7.
326 Several solid phases were detected by XRD in cultures grown for 17 days at elevated metal
327 concentrations. Abiotic samples showed no formation of specific crystalline solid phases, with
328 the exception of calcite in one sample. Precipitates analyzed from the *Penicillium* cultures grown
329 with $CuSO_4$ were identified as Cu-oxalate (moolooite) and, in some cases, vivianite
330 ($Fe_3(PO_4)_2 \cdot 8H_2O$). Cultures grown in a cadmium medium also formed Cd-oxalate as crystallized
331 solid phase. Oxalic acid is produced by a wide variety of fungi (for review, see Gadd et al., 2014)
332 and mycogenic metal oxalate formation has been demonstrated as an important example of
333 biomineralization (Baldrian, 2003; Fomina et al., 2005; Burford et al., 2006; Gadd, 2007., Gadd,
334 2010; Fomina et al., 2010). The mineral moolooite was first discovered near a sulfide-bearing
335 quartz outcrop at near the Mooloo Creek in Western Australia, in 1977 (Clarke & Williams,
336 1986). On the basis of the sample location, the authors suggested that the mineral was formed by
337 interaction between solutions derived from bird guano and weathering copper sulfides. Our
338 finding of moolooite formation by copper-resistant *Penicillium* spp. and other reports of
339 moolooite formation by fungi (references), suggests that the bird guano might supply an N- and
340 P-rich organic substrate for fungal growth, which was responsible for the Cu-oxalate formation.
341 Retgersite ($NiSO_4 \cdot 6H_2O$) was formed in mycelia when *Penicillium* ShG4Cwas cultivated in a
342 nickel chloride-dosed medium. Retgersite is a common secondary mineral associated with mine
343 drainage. Bieberite ($CoSO_4.7H_2O$), a poorly soluble, hydrated Co-sulfate, was detected in
344 precipitates from cobalt-enriched cultivations. Based on transmission electron micrographs,
345 bieberite crystals were associated with mycelia. To our knowledge, biomineralization of these
346 minerals has not hitherto been reported. Some fungi, including *Penicillium*, are able to oxidize
347 sulfur to form SO_4^{2-} (Sterflinger, 2000), which may play a role in the observed formation of
348 insoluble sulfates of cobalt and nickel.
349

CONCLUSION

The results of this study indicate that metal- and acid-tolerant *Penicillium* survive in mining environments with extremely low pH and high metal / metalloid concentrations and, moreover, that they may be involved in biogeochemical transformations of copper, cadmium, nickel, and cobalt in these ecosystems. Strains ShG4B and ShG4C of *Penicillium*, isolated from mine waste leachate at Sherlovaya Gora polymetallic sulfide mine (Transbaikalia, Siberia, Russia) may have some potential in bioremediation, due to their ability to immobilise toxic metal ions in solid phases; they may also have potential in metal bioleaching due to their production of organic acids. The mechanisms allowing the two novel strains of *Penicillium* to tolerate metals are still to be elucidated. A transcriptomic analysis is underway to shed light on the high copper and arsenic resistance in ShG4C.

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Figure captions

Figure 1. (A) Location map. The rectangle on the map shows the location of the study area within the Russian Federation. (B) The stockpile near the tailings area, with the white arrow showing the location where the sediment sample was collected. (C) The puddle where the water sample for chemical analysis and sediment sample for fungal isolation have been collected.

Figure 2. Elemental analysis of water sample and XRD analysis of the sediment sample from site ShG4. Letter codes: Ba = barysilite, $Pb_8Mn^{2+}[Si_2O_7]_3$, (PDF-20-0714), Mz = magnesiozippeite, $Mg[(UO_2)_2O_2(SO_4)](H_2O)_{3.5}$, (PDF-29-0876), Rd = renardite, $Pb(UO_2)_4(PO_4)_2(OH)_4{\cdot}7H_2O$, (PDF-11-0215), Sk = sklodowskite, $Mg(UO_2)_2(HSiO_4)_2{\cdot}5H_2O$, (PDF-08-0447), Va = vanuralite, $Al(UO_2)_2(VO_4)_2(OH){\cdot}11(H_2O)$, (PDF-15-0506),Qu = quartz, SiO_2, (PDF-33-1161).

Figure 3. Mycelium (A) and conidiophores (C) of ShG4B and mycelium (B) and conidiophores (D) of ShG4C, grown on potato dextrose agar (1), modified Czapek agar (2), and malt extract agar (3) after 5 days of incubation standardized to initial pH of 4.6. Hyphal morphology and cleistothecia of isolate ShG4C grown for 5 days at initial pH 2.5 in the presence of 3 g Cu l^{-1} (E) and 0.2 g Cd l^{-1} (F).

Figure 4. Phylogenetic analysis of concatenate sequences of genes RPB1, RPB2, Cct8 and Tsr1 with maximum likelihood, which was made with MEGA6 software (Tamura et al. 2013). Concatenation was based on Houbraken and Samson (2011).

Figure 5. Colony radius (A) and biomass production (B) by the two *Penicillium* isolates as a function of the initial pH. The cultures were incubated for 17 days before colony radius/biomass was measured. Vertical bars = standard error (n = 5). Final pH in the culture medium is shown by open circles (ShG4B) and squares (ShG4C) on 5B.

Figure 6. Development of pH with time in the ShG4B and ShG4C cultures. Vertical bars = standard error (n = 3).

Figure 7. XRD analysis of *Penicillium* ShG4C mycelia grown in modified Czapek media in the

absence of metals (1) and in the presence of 3 g l^{-1} (2) Co, (3) Ni, (4) Cd, and (5) Cu. Letter codes: Bi = bieberite (PDF-16-0487), Mo = moolooite (PDF-21-0297), Ox = Cd-oxalate (PDF-14-0712), Re = retgersite (PDF-47-1811), Vi = vivianite (PDF-03-0070).

Figure 8. XRD analysis of heat-killed mycelia of ShG4C cultivated for 17 days with 3 g l^{-1} (1) Co, (2) Ni, (3) Cd, and (4) Cu. Letter codes: Mo = moolooite, Vi = vivianite.

Figure 9. Scanning electron micrographs of ShG4C mycelia grown in the presence of 3 g Cu l^{-1}. (A) mycelia after 20 days of incubation, with more details shown in (B) and (C). (D) Mycelia after 40 days of incubation, with more details shown in (E).

Figure 11. Scanning electron micrographs of ShG4C mycelia grown for 20 days in the absence of metal (A) and in the presence of 3 g Co l^{-1} (B and C).

Figure S1. XRD patterns of chemical controls for the test metals (3 g l^{-1} each) after 17 days of contact time, prepared in the absence of biomass. (1) Co; (2) Ni; (3) Cd; and (4) Cu.

Figure S2. Scanning electron micrographs (A) and (B) of ShG4C mycelia grown in the presence of 3 g Cu l^{-1} for 20 days. The corresponding EDXA results of (A) and (B) mycelial samples are also shown, indicating elevated levels of Cu in mycelia and extracellular vesicle. The bars indicate standard errors (n=6).

Figure S3. Scanning electron micrographs of ShG4C mycelia grown in the presence of 3 g Co l^{-1} for 20 days. (A) a cluster of Co-sulfate crystals; (B), mycelial fragment. The corresponding EDXA results for (A) and (B) indicate elevated levels of Co in the cluster of crystals especially.

Figure S4. Scanning electron micrographs (A) and (B) of ShG4C mycelia grown in the presence of 3 g Cd l^{-1} for 20 days. Cd was not detected in the EDXA of mycelial sample shown in micrograph (B).

Table 1 - PCR primers used in the study

Locus	Primer	Sequence (5'-3')	Annealing (T°C)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	55	Ying et al., (2012)
	1492R	GGTTACCTTGTTACGACTT		
18S rRNA	EukA	AACCTGGTTGATCCTGCCAGT	55	Behnke et al., (2006)
	EukB	TGATCCTTCTGCAGGTTCACCTAC		
	Euk360F	CGGAGA(A/G)GG(A/C)GC(A/C)TGAGA		
ITS	ITS1	TCCGTAGGTGAACCTTGCGG	55	(Viktor et al., 2013)
	ITS4	TCCTCCGCTTATTGATATGC		
Tsr1	F1526Pc	GARTAYCCBCARTCNGAGATGT	52	(Houbraken & Samson, 2011)
	R2434	ASAGYTGVARDGCCTTRAACCA		
Cct8	F94	CGCAACAAGATYGTBATYAACCA	48	(Houbraken & Samson, 2011)
	R1595	RTCMACRCCNGTIGTCCAGTA		
RPB1	F1843	ATTTYGAYGGTGAYGARATGAAC	52	(Houbraken & Samson, 2011)
	R3096	GRACRGTDCCRTCATAYTTRACC		
RPB2	5F_Eur	GAYGAYCGKGAYCAYTTCGG	52	(Houbraken & Samson, 2011)
	7CR_Eur	CCCATRGCYTGYTTRCCCAT		

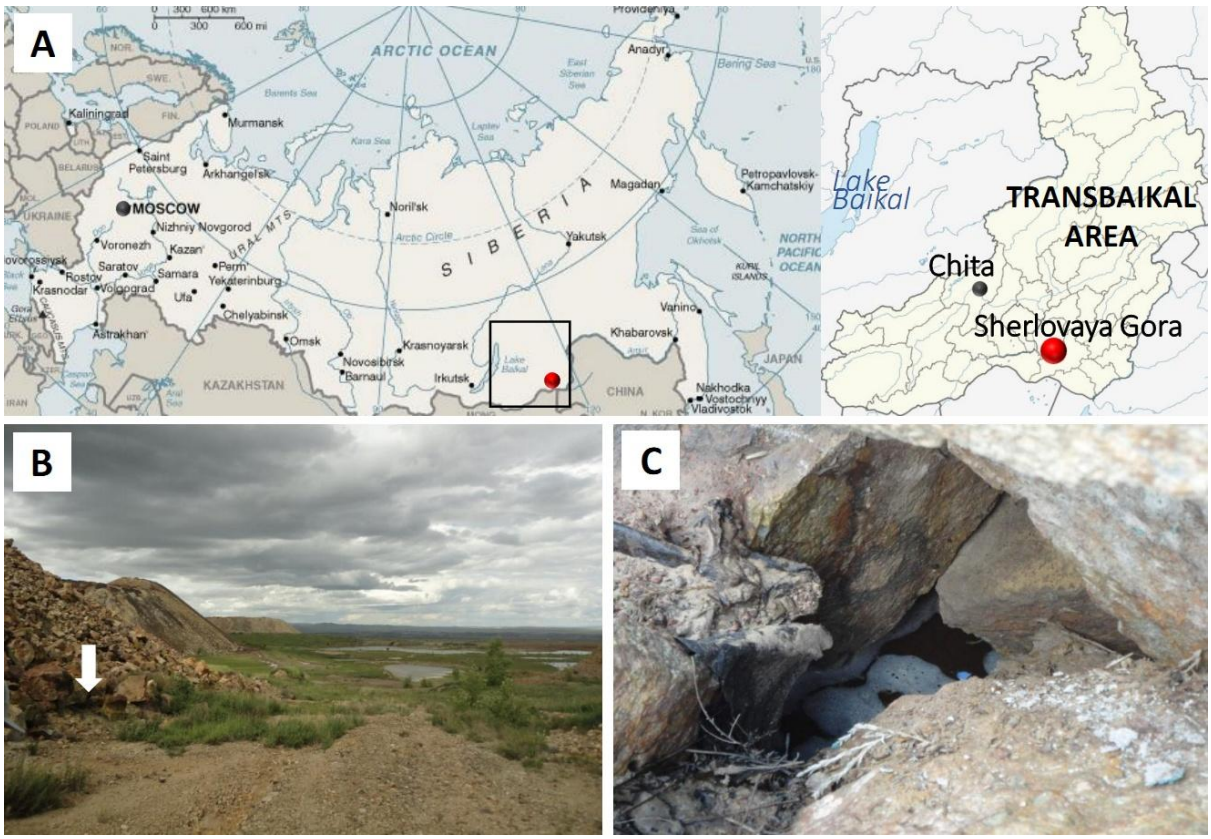

Figure 1. Site location. A, Sherlovaya Gora on the map of Russian Federation. B, Place of sampling on the ground. C, Superficies of the sampling site.

Element	Concentration, mg·L^{-1}	Element	Concentration, mg·L^{-1}	Element	Concentration, mg·L^{-1}	Element	Concentration, mg·L^{-1}
1	2	1	2	1	2	1	2
Halogens		Cr	7.23	**REE**			
Br	0.083	Cs	0.023	Y	23.1	Gd	3.11
I	0.0024	Ga	0.084	Sc	1.15	Tb	0.74
Cations and main metals		Ge	0.036	La	0.78	Dy	5.13
Ca	500	Hf	0.021	Ce	3.83	Ho	0.98
Mg	1137	In	2.10	Pr	0.62	Er	2.92
Na	0.35	Li	2.98	Nd	3.16	Tm	0.47
K	0.043	Mo	0.027	Sm	1.70	Yb	2.744
Al	2672	Nb	0.35	Eu	0.046	Lu	0.41
Fe	18316	Ni	44.0				
Mn	498	Pb	31.7				
Cu	2083	Rb	0.038				
Zn	2755	Sb	3.10				
Si и P		Se	6.02				
P	51.5	Sn	4.00				
Si	51.7	Sr	0.24				
Minor and Trace Elements		Ta	0.0069				
Ag	0.00663	Te	0.012				
As	2910	Th	6.37				
Ba	0.0021	Ti	2.92				
Be	0.083	Tl	0.0026				
Bi	2.27	U	9.16				
Cd	55.7	V	3.24				
Co	12.73	W	0.15				

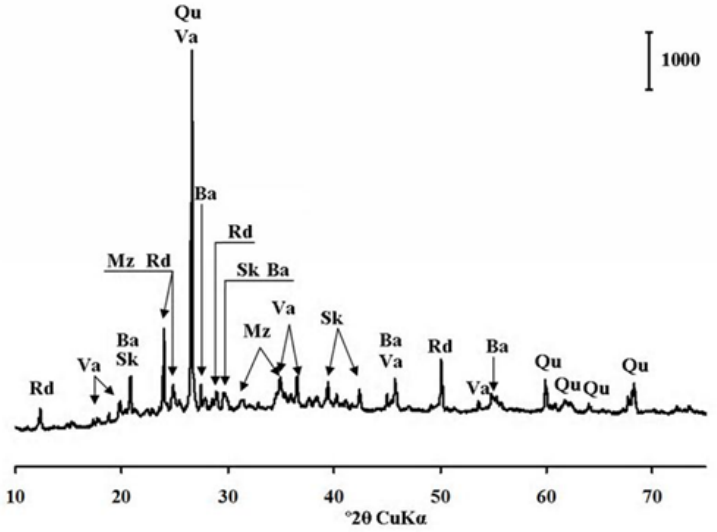

Figure 2. Physicochemical characteristics of mining wasters. In the table shown an elemental analysis (ICP-MS) of the waters samples from site ShG4. Graph - XRD analysis of the sediment sample from site ShG4. Letter codes: Ba = barysilite, $Pb_8Mn^{2+}[Si_2O_7]_3$ (PDF-20-0714), Mz = magnesiozippeite, $Mg[(UO_2)_2O_2(SO_4)](H_2O)_{3.5}$, (PDF-29-0876), Rd = renardite, $Pb(UO_2)_4(PO_4)_2(OH)_4{\cdot}7H_2O$, (PDF-11-0215), Sk = sklodowskite, $Mg(UO_2)_2(HSiO_4)_2{\cdot}5H_2O$, (PDF-08-0447), Va = vanuralite, $Al(UO_2)_2(VO_4)_2(OH){\cdot}11(H_2O)$, (PDF-15-0506), Qu = quartz, SiO_2, (PDF-33-1161).

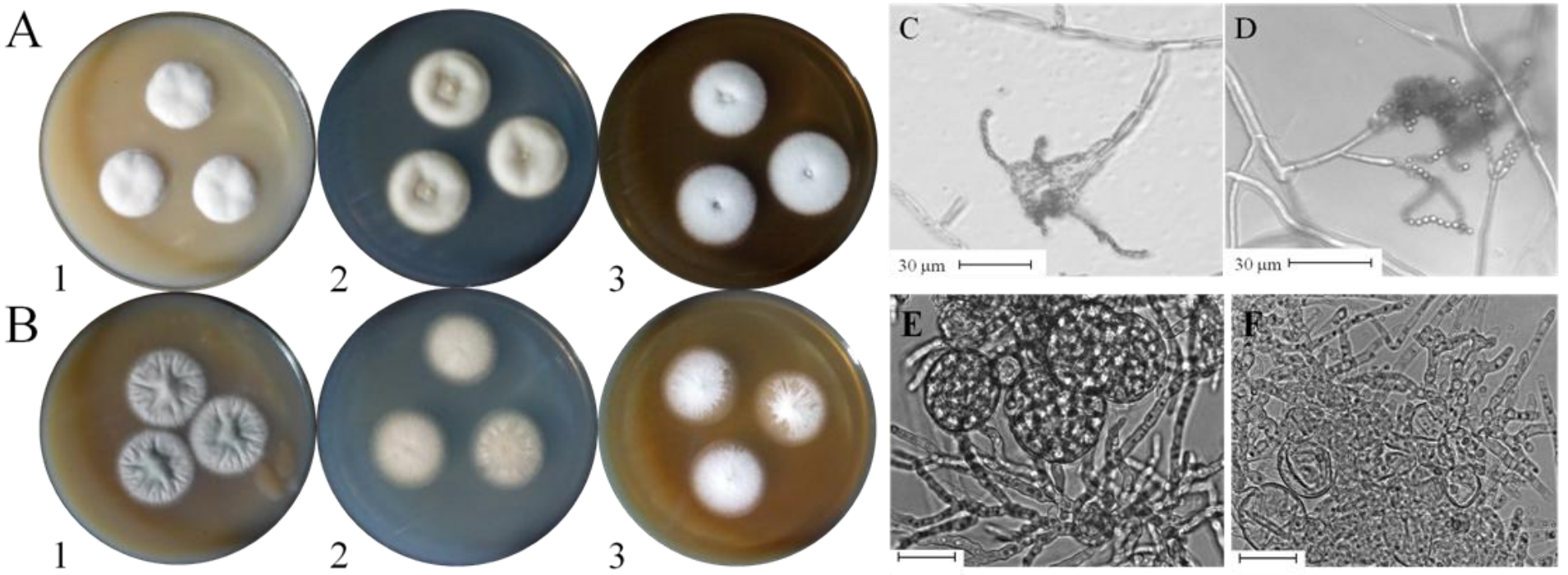

Figure 3. Morphological description of fungal isolates. Colonies ShG4B (A) and ShG4C (B) after 5 days of incubation on solid media standardized to initial pH 4.6. 1, Potato dextrose agar; 2, modified Czapek agar; 3, malt extract agar. Mycelium and conidiophores of ShG4B (C) and ShG4C (D) on modified Czapek agar. Hyphal morphology and cleistothecia of isolate ShG4C grown for 5 days at initial pH 2.5 in the presence of (E) 3 g Cu L^{-1} and (F) 0.2 g Cd L^{-1}. The bar = 20 µm.

Table 2. Maximum arsenic and metal ion concentration allowing strain ShG4B and ShG4C growth.

Isolate	Concentration of element (g L^{-1})					
	As^{5+}	Cd^{2+}	Co^{2+}	Cu^{2+}	Ni^{2+}	Pb^{2+}
ShG4B	4	1	3	4	1	1
ShG4C	6	10	2	10	0.5	1

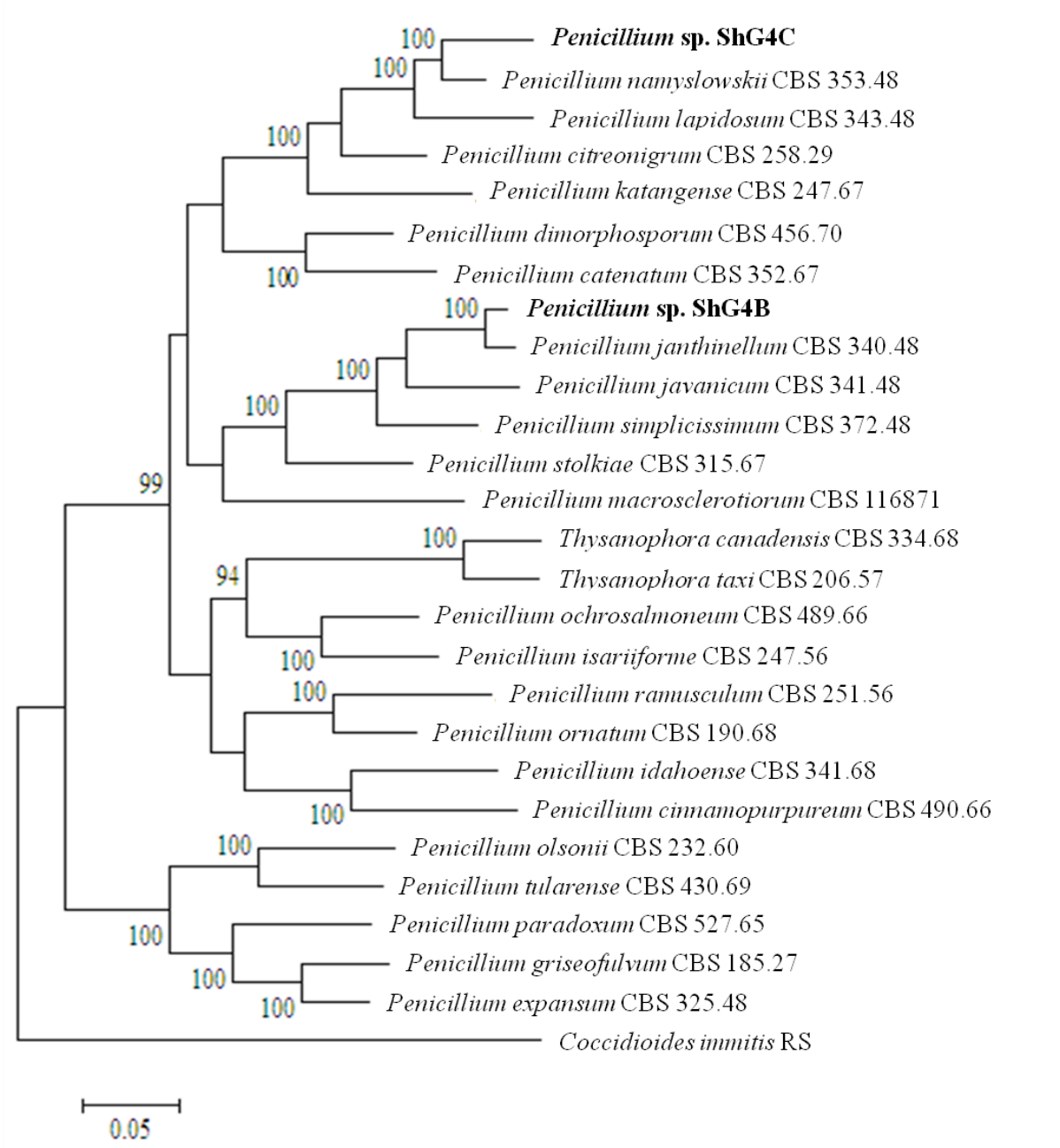

Figure 4. Phylogenetic analysis of concatenate sequences of genes RPB1, RPB2, Cct8 and Tsr1 with maximum likelihood, which was made with MEGA6 software (Tamura et al. 2013). Concatenation was based on Houbraken and Samson (2011).

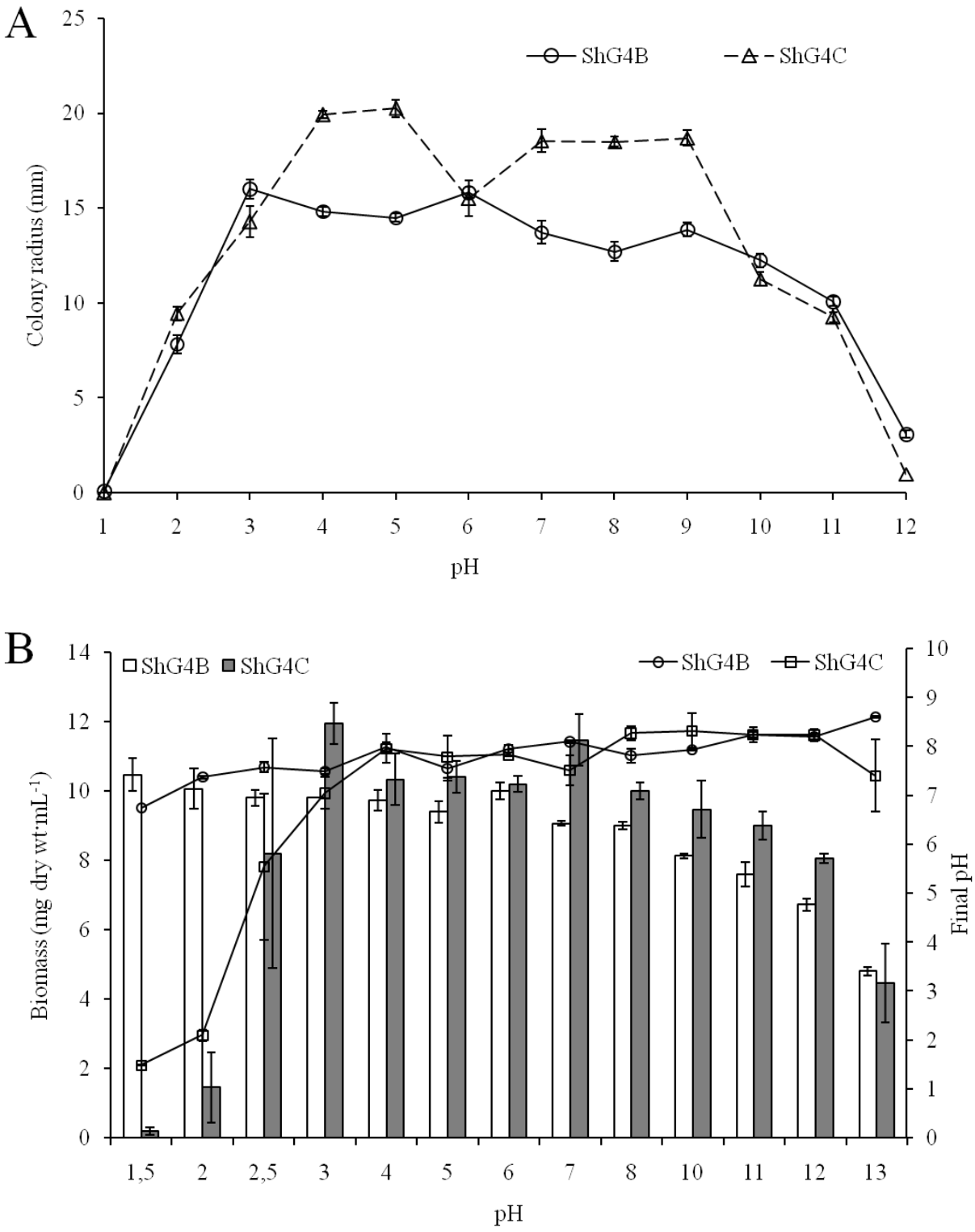

Figure 5. Growth of the two *Penicillium* isolates as a function of the initial pH. (A) The colony radius on solid Czapek medium was measured on the 6th day (n = 12). (B) The liquid cultures were incubated for 17 days before biomass was measured. The inoculums size was 10 mg. (The common data of two independence experiment, *n* = 10). The final pH was measured on the 17th day (n = 3). Vertical bars = standard error.

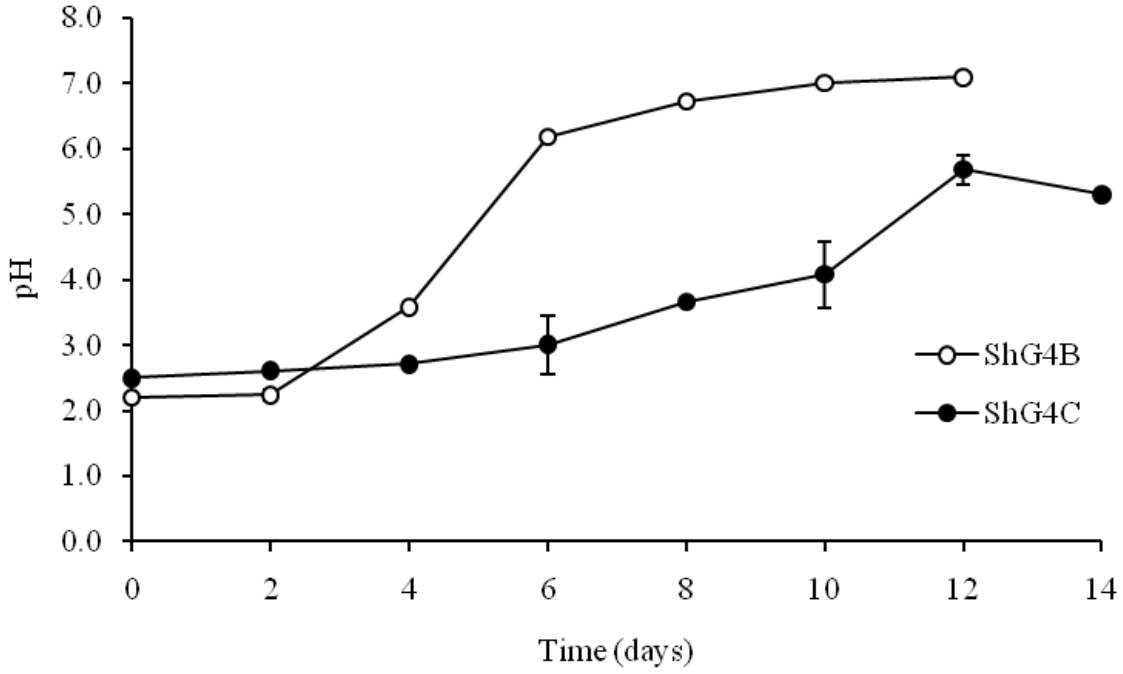

Figure 6. Time course of pH changes in the ShG4B and ShG4C cultures. The inoculums size was 30 mg. Vertical bars = standard error (n = 3).

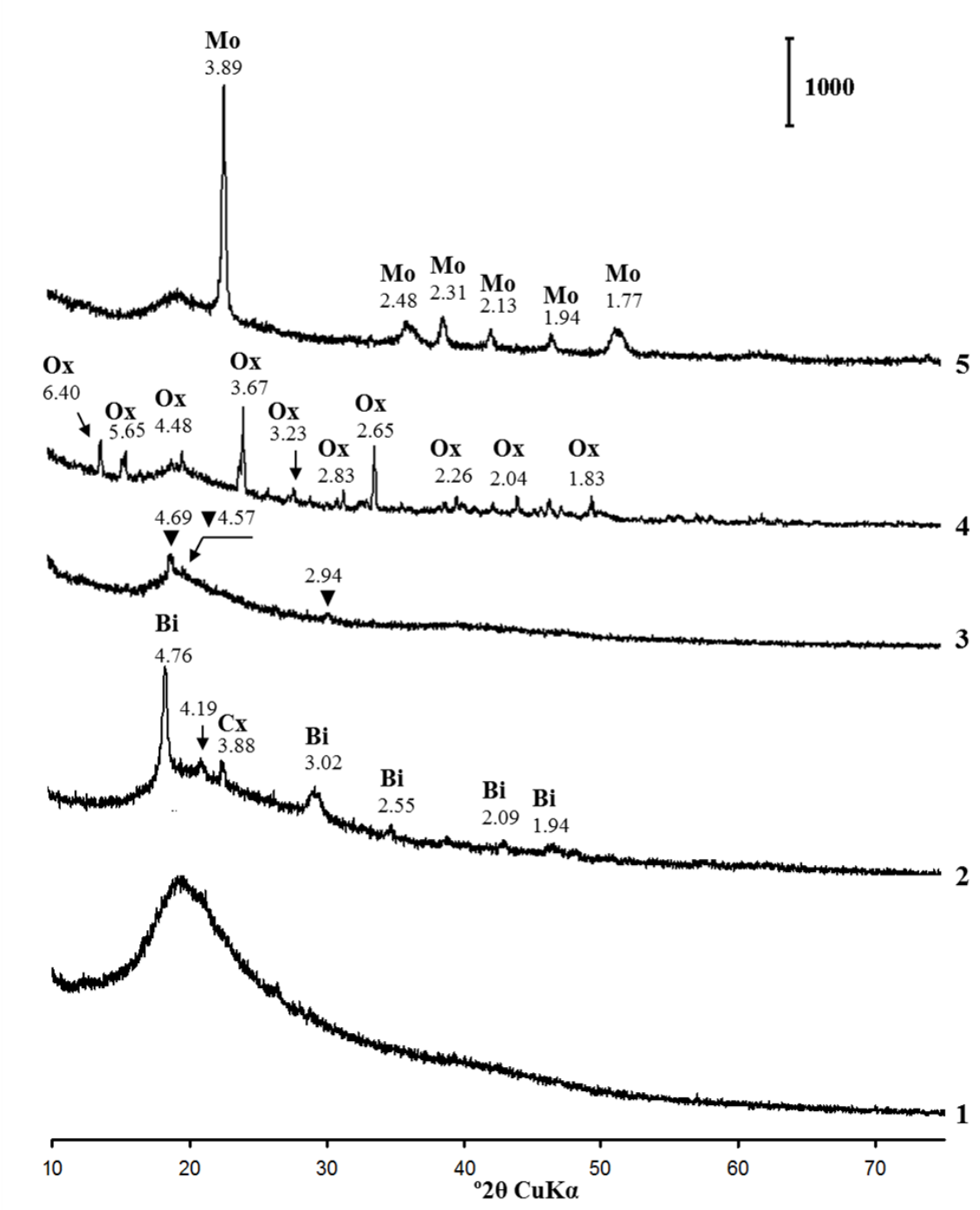

Figure 7. XRD analysis of *Penicillium* ShG4C mycelia grown in modified Czapek media in the absence of metals (1) and in the presence of 3 g L^{-1} (2) Co, (3) Ni, (4) Cd, and (5) Cu. Letter codes: Bi = bieberite (PDF-16-0487), Cx = Co-oxalate (PDF-37-0719), Mo = moolooite (PDF-21-0297), Ox = Cd-oxalate (PDF-14-0712), ▼ = retgersite (PDF-47-1811) or morenosite (PDF-01-0403).

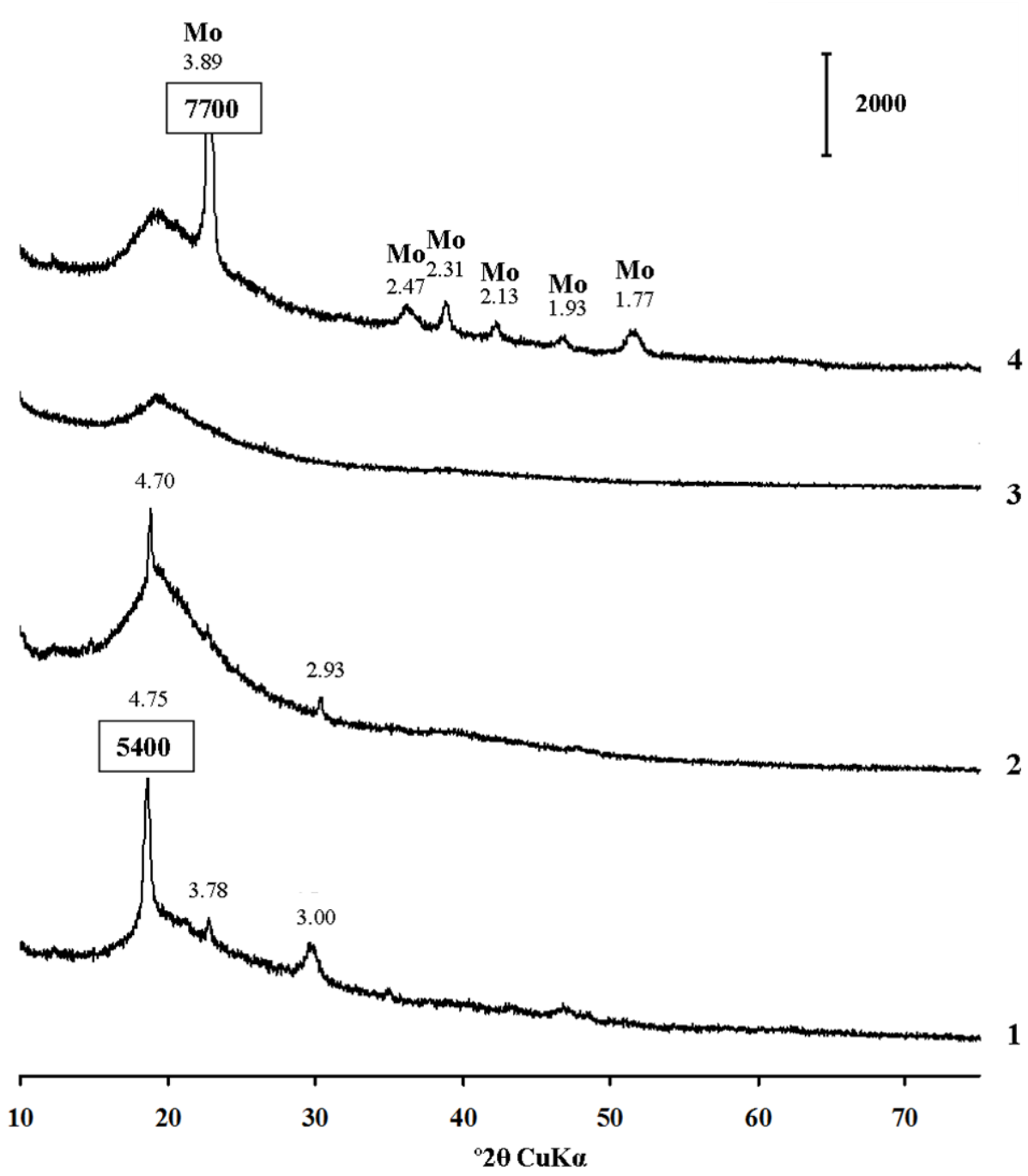

Figure 8. XRD analysis of heat killed mycelia of ShG4C contacted for 17 days with 3 g L^{-1} (1) Co, (2) Ni, (3) Cd, and (4) Cu. Letter codes: Mo = moolooite.

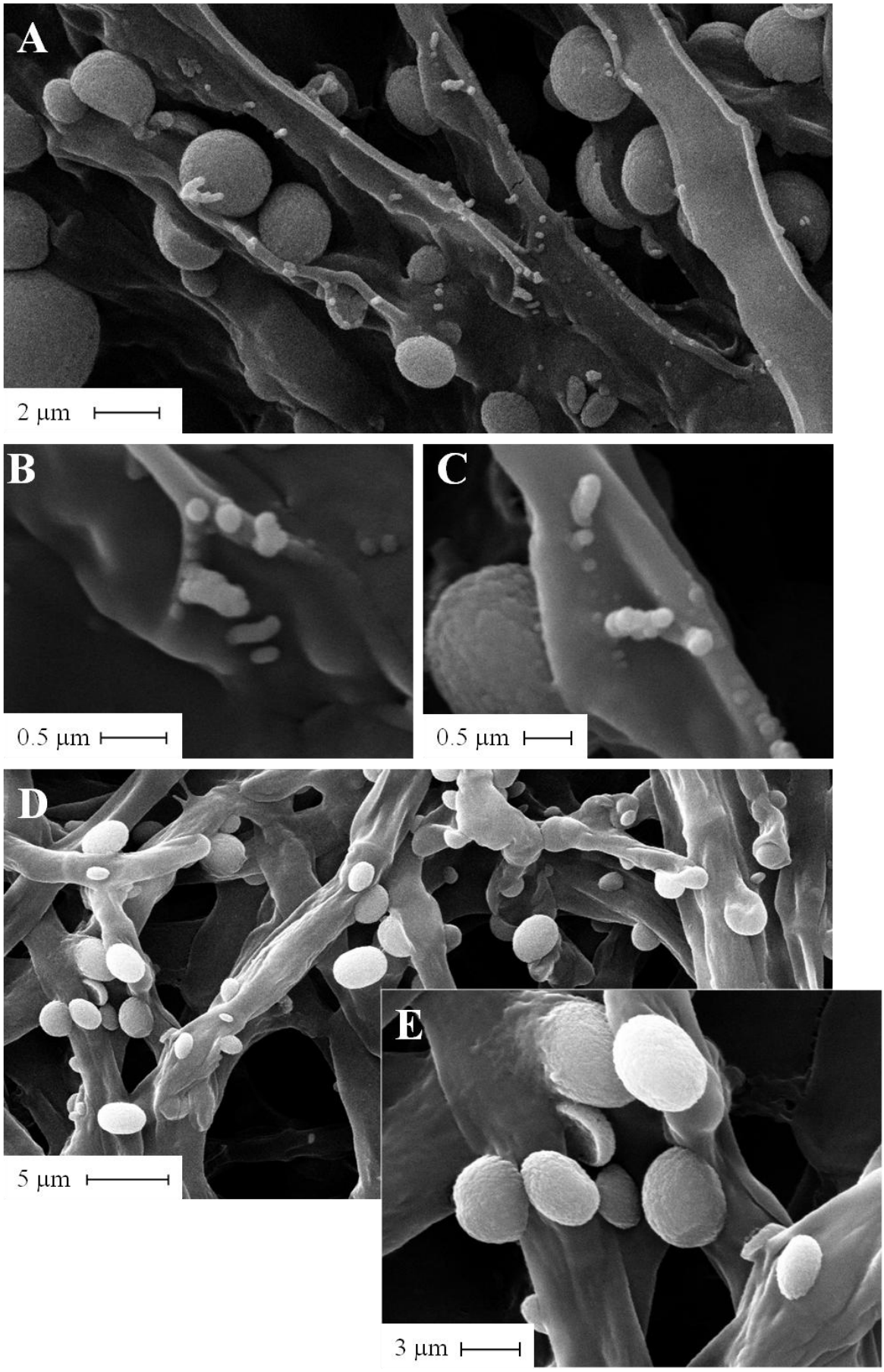

Figure 9. Scanning electron micrographs of ShG4C mycelia grown in the presence of 3 g Cu L^{-1}. A, mycelia after 20 days of incubation, with more details shown in B and C. D, mycelia after 40 days of incubation, with more details shown in E.

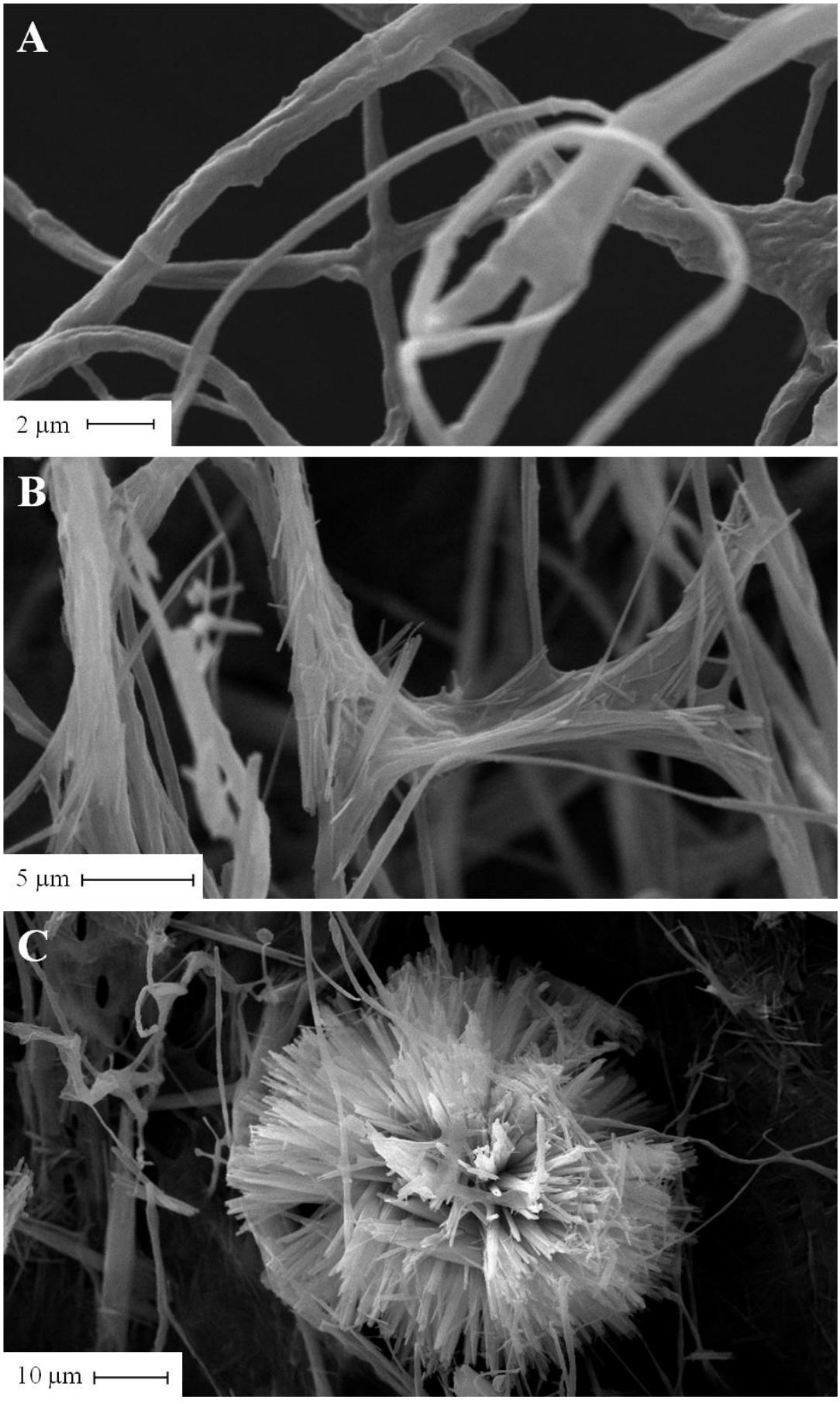

Figure 10. Scanning electron micrographs of ShG4C mycelia grown for 20 days (A) in the absence of metal and (B and C) in the presence of 3 g Co L^{-1}.

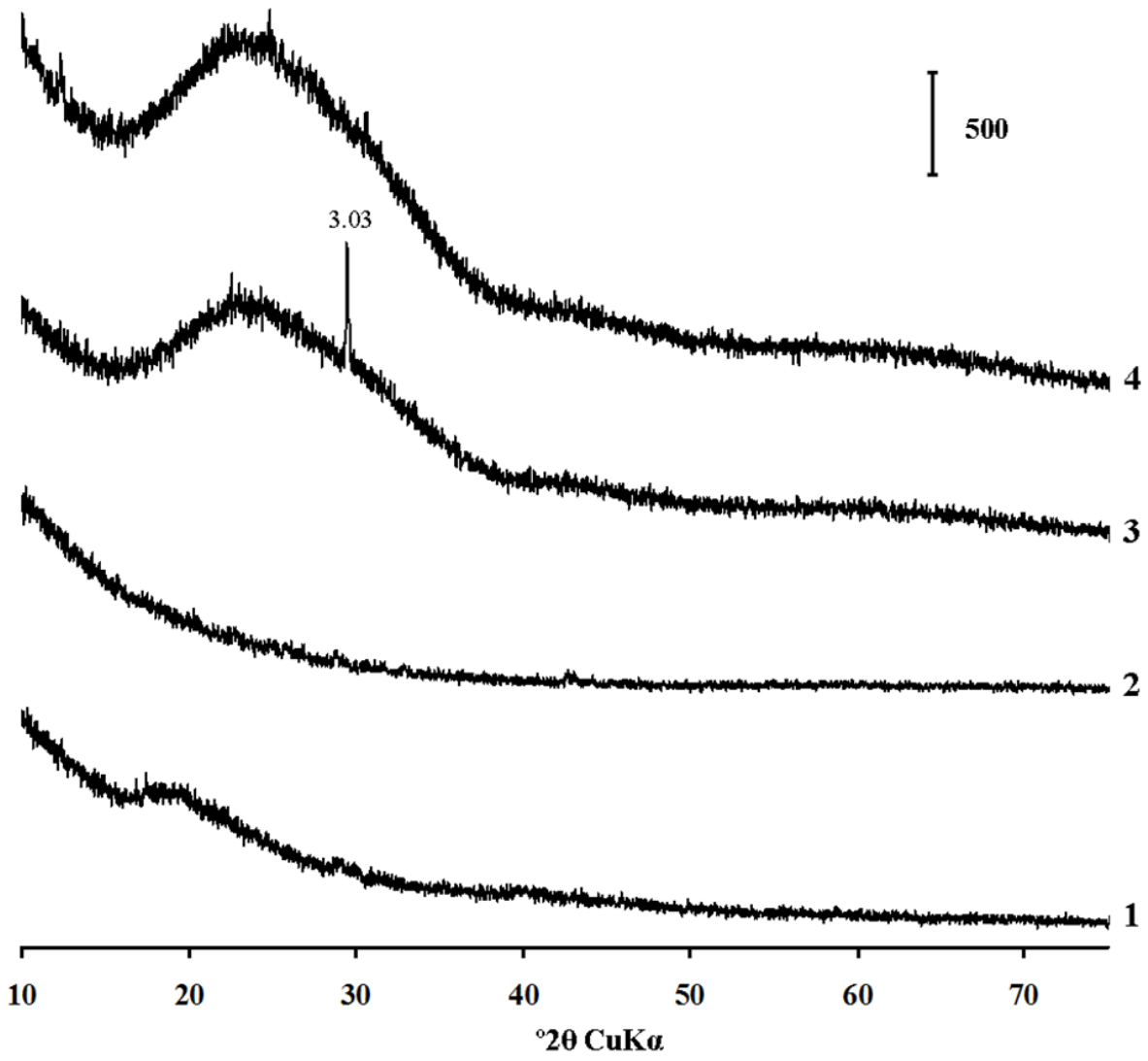

Figure S1. XRD patterns of chemical controls for the test metals (3 g L^{-1} each) after 17 days of contact time, prepared in the absence of biomass. 1, Co; 2, Ni; 3, Cd; and 4, Cu.

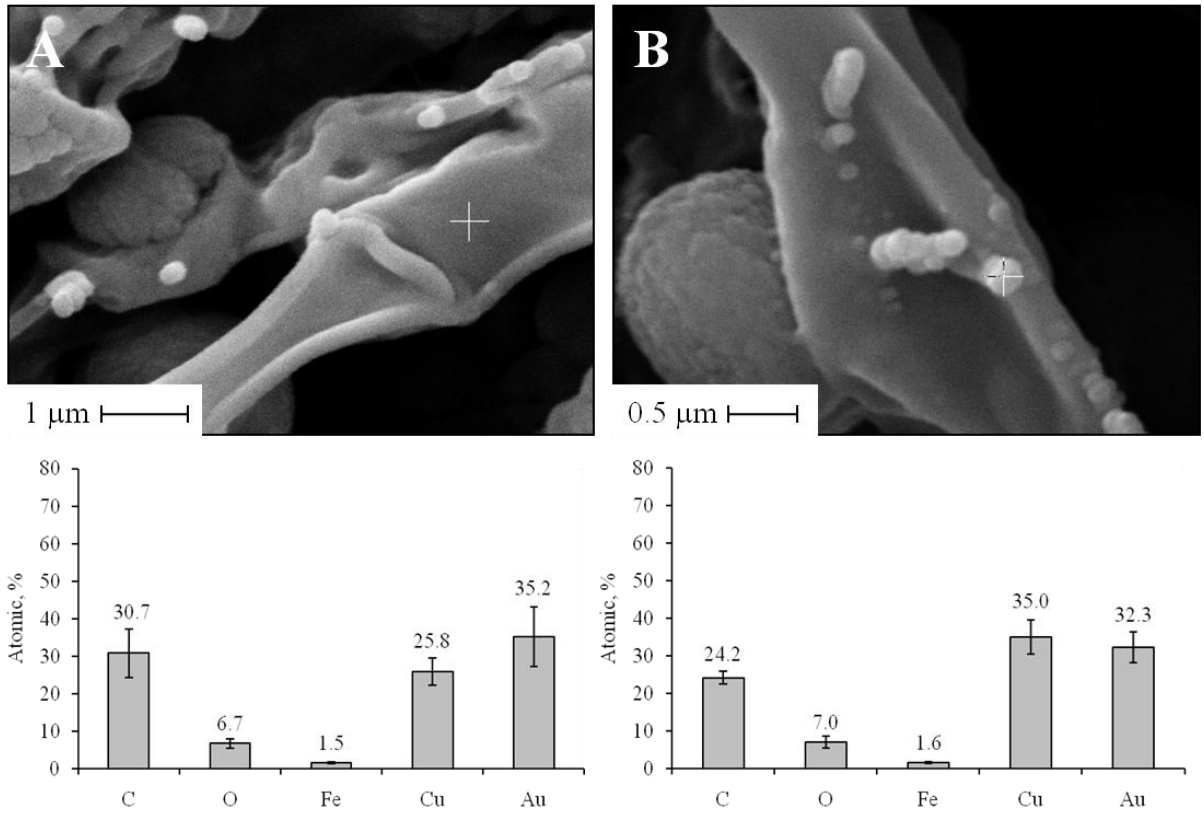

Figure S2. Scanning electron micrographs (A and B) of ShG4C mycelia grown in the presence of 3 g Cu L^{-1} for 20 days. The corresponding EDXA results of A and B mycelial samples are also shown, indicating elevated level of Cu in mycelia and extracellular vesicle. The bars on graphs indicate standard errors (n=6).

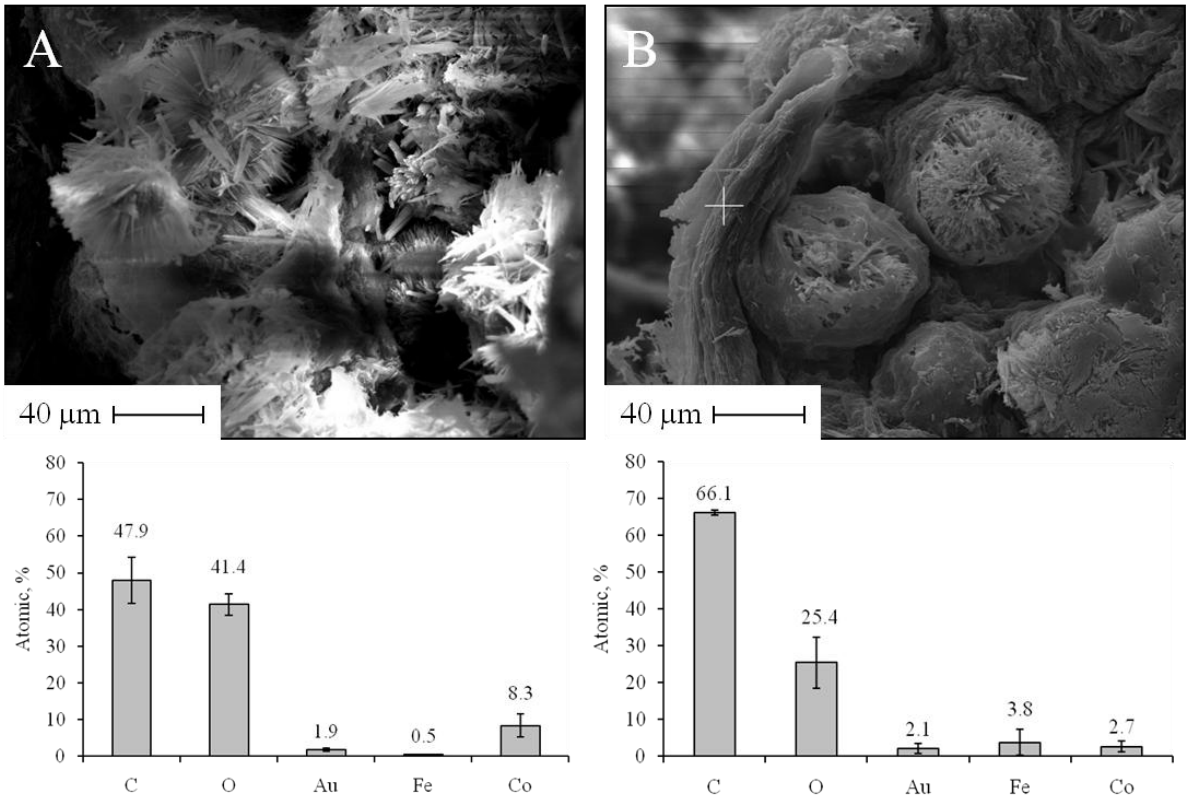

Figure S3. Scanning electron micrographs of ShG4C mycelia grown in the presence of 3 g Co L^{-1} for 20 days. A, a cluster of Co-oxalate crystals, B, mycelial fragment. The corresponding EDXA results for A and B indicate elevated level of Co in the cluster of crystals especially. The bars on graphs indicate standard errors (n=6).

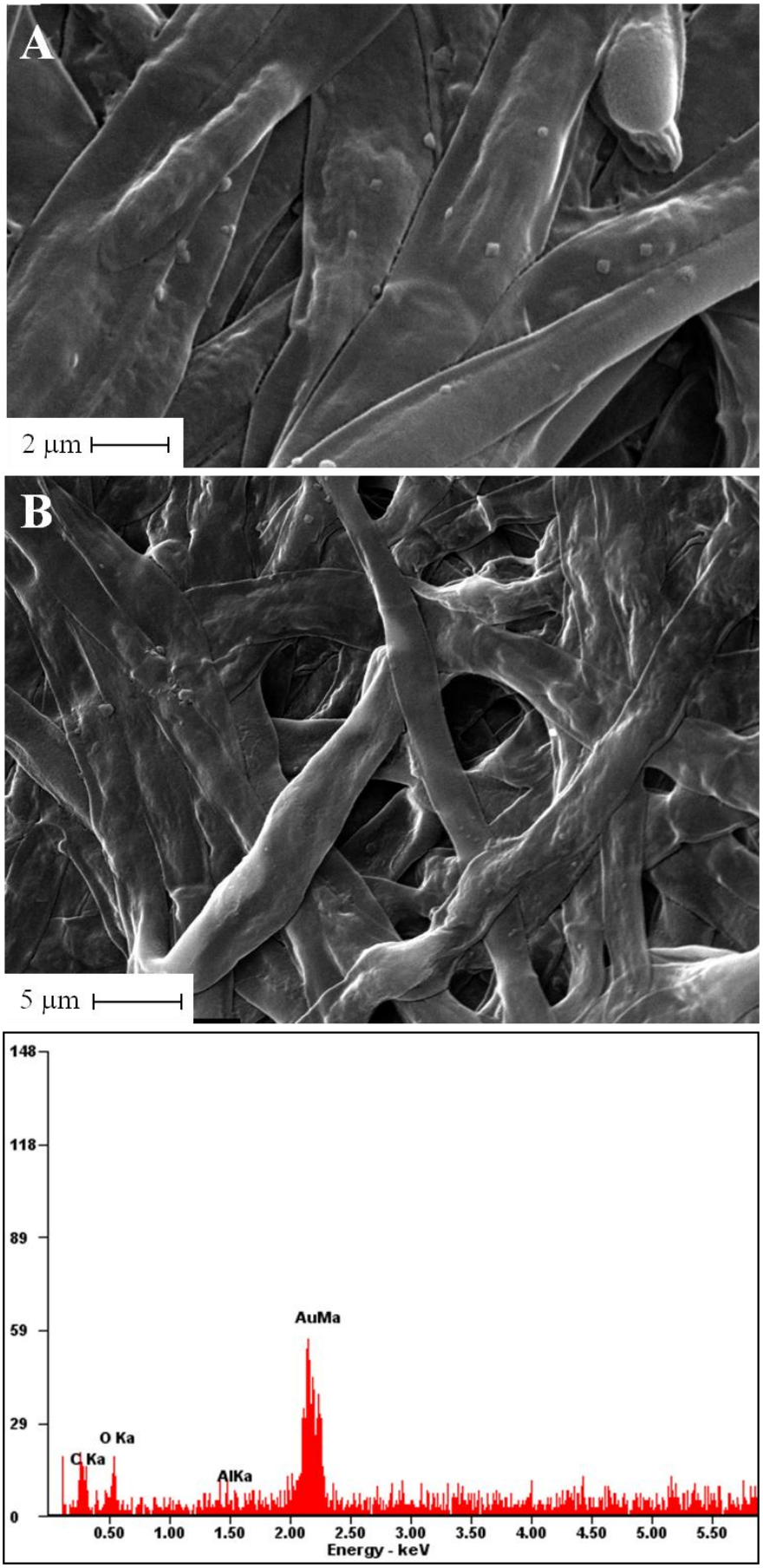

Figure S4. Scanning electron micrographs (A and B) of ShG4C mycelia grown in the presence of 3 g Cd L^{-1} for 20 days. Cd was not detected in the EDXA of mycelial sample shown in micrograph B.